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### EVALUATION OF AN INEXPENSIVE FIELD TEST FOR RULING OUT THE PRESENCE OF BIOLOGICAL THREAT AGENTS IN SUSPICIOUS POWDERS

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## PREFACE

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# EVALUATION OF AN INEXPENSIVE FIELD TEST FOR RULING OUT THE PRESENCE OF BIOLOGICAL THREAT AGENTS IN SUSPICIOUS POWDERS

## 1. INTRODUCTION

In October 2001, the presence of *Bacillus anthracis* was confirmed in a number of letters to Senate offices and news agencies. Since then, first responders/hazardous materials technicians (HAZMAT) are frequently called to respond to incidents involving suspicious white powders, most of which are hoaxes. In the absence of effective, efficient, validated field pre-screening tools, responders must rely on public health laboratories to analyze the samples before resolving the incident. The laboratory analyses can be costly and time-consuming, disrupting normal facility operations and consuming scarce testing resources. In addition, the capacity of public health laboratories is limited and preventing overload of these laboratories is critical. The ability to easily and reliably "triage" suspicious powders in the field could lower cost and decrease response time in addition to allowing analytical laboratories to focus their efforts on high priority samples.

Although a number of field pre-screening technologies for biological agents are commercially available, most have not been properly tested or validated. As a result, the responder community does not have access to reliable, credible information regarding the proper use and limitations of these pre-screening technologies. In addition, the public health community is alarmed that first responders may rely on inaccurate results obtained from these technologies to make decisions that could affect public health. Validated performance, appropriate protocols and policies, as well as training are all critical to the successful use of pre-screening tools.

After the mailing of anthrax laced letters, the Department of Homeland Security (DHS) and the General Services Administration (GSA) sought to explore cost-effective methods for first responders to use to handle and pre-screen suspicious white powder samples. In December 2002, a technical working group was assembled at the Center for Domestic Preparedness (CDP), "to develop a science-and consensus based protocol to support emergency response personnel at events involving suspect "anthrax" letters or packages."<sup>1</sup> The working group consisted of experienced members of the scientific community and senior practitioners from the fire service, law enforcement, emergency medical, defense, hazardous materials and responder-education fields. The working group's efforts resulted in the design of an economical five-step pre-screening method that was published in 2003 in *Homeland First Response*.<sup>1</sup> The purpose of the method is to enable first responders to rule out the presence of biological agents in suspicious powders in a timely, cost effective manner thus reducing the need for tests such as PCR or hand held assays. The proposed five-step method focuses on measuring a few key physical and chemical properties of a powder (i.e., appearance, solubility in water, acidity, protein content, etc.) to determine

<sup>1</sup>Franz, D., et al. Suspicious White Powder. *Homeland First Response*. July/August 2003; 26-31.

the potential of a suspicious white powder to be a biological threat. The method is designed only to rule out the presence of a biological threat agent. It cannot be used to confirm the presence or identity of a specific biological agent. To keep costs at a minimum, the five-step method proposes the use of commercially available urinalysis strips to detect protein content and pH test strips for measuring acidity and basicity. The following study presents the findings of laboratory and field tests conducted on the five-step method to evaluate its actual effectiveness in screening powdered substances. The study includes a survey of commercially available components, pH and urine test strips, which could be used to perform the five-step method in a cost effective manner. Two phases of testing were conducted; the first phase in the laboratory and the second in the field. In the initial laboratory study, six different protein strips were evaluated against two biological agents and fourteen different commonly used hoax materials. In the follow on field study, only two protein strips were evaluated against the fourteen hoax materials (biological threat agents were not used due to safety constraints at the site). In the field study, HAZMAT technicians were used to identify factors that might affect performance in the field such as personal protective equipment or training.

## 2. THE FIVE-STEP METHOD

Table 1 outlines the steps of the five-step method and how the results from each step should be interpreted with regards to the presence of a biological agent.

Table 1. Proposed Biological Field Test System

Test To Be Conducted	Possibly, a Biological (or of respirable size)*	Not Likely to be Biological (or not of respirable size)*
<b>Step 1)</b> Collect two samples: Sample 1 for reference laboratory analysis** ; a volume equivalent to one restaurant sugar packet (ca. 1 gm) or more, if available. Sample 2 for field analysis; a volume similar to the size of a small pea or kernel of corn.		
<b>*Step 2)</b> Place material to be analyzed in a dry ca. 3ml clean glass vial and secure lid. Shake vigorously for a few seconds and observe.	Fine cloud or haze hangs above sample for several seconds after shaking is stopped	All material falls to bottom of vial, like salt in a salt shaker, after shaking; air above material is clear
<b>Step 3)</b> Remove lid, fill vial ca. two-thirds (ca. 2ml) with distilled water and reseal lid. Shake vigorously for 15 seconds and observe.	Sample appears to mix with water, but does not dissolve. Liquid contents remain turbid or cloudy.	Sample dissolves in water and becomes clear with or without larger particles settling to the bottom.
<b>Step 4)</b> Remove lid of vial and dip pH test strip into water. Remove strip, wait 30 seconds and read result on pH strip container.	pH between 5 and 9	pH less than 5 or greater than 9
<b>Step 5)</b> Remove lid of vial and dip one protein test strip. Remove strip, wait 30 seconds and read result on protein strip container.	Protein is present.	Protein is not present.

**NOTE:** The shaking of dry powder in vial as described in step 2 provides only an indication of particle size. This part of the test protocol does not provide an indication regarding the potential for the material being of biological origin.

\*\* Standing procedures that address packaging, chain of custody and decontamination for suspect samples should be established with the local FBI in advance of an event.

### 3. EXPLANATION OF THE FIVE STEPS

#### 3.1 Step 1. Sample Collection.

The available powder is split into two portions. The majority of the sample should be collected for analysis by a Laboratory Response Network (LRN) laboratory. A smaller portion, the volume similar to the size of a pea or kernel of corn, should be collected for field analysis with the five-step method. If there is insufficient sample to provide the LRN with the required portion, then the first responder should not conduct the five-step test and should package the sample for immediate dispatch to the LRN for analysis.

#### 3.2 Step 2. Particle Size.

At this step, close observation may reveal if the powder has been mixed either with a fluidizer or carrier substance or if the powder has small particles of respirable size. Respirable powders are more effectively breathed in by a victim and may form a fine cloud or haze in the bottle or stick to the sides of the vial. In contrast, a non-respirable powder may fall to the bottom of the vial. This step was intended to only provide information on the inhalational threat and does not provide an indication of the presence of a biological agent.

#### 3.3 Step 3. Solubility/Turbidity.

If the powder contains a biological agent, it is likely that the material will not completely dissolve in water, and the solution will appear turbid. In contrast, many of the hoax powders are water-soluble, and upon the addition of water to the vial, the material will dissolve with undissolved particles falling to the bottom of the vial, and the water will become clear.

#### 3.4 Step 4. pH Analysis.

In this step, the acidity/basicity of the solution are determined using commercially available pH test strips. Biological material traditionally is stable at pH values between 5 and 9. In contrast, some hoax powders may have pH values that are more acidic (i.e., have pH values <5) or more basic (i.e., have pH values >9).

#### 3.5 Step 5. Protein Content.

Biological threat agents (including bacteria, toxins, and viruses) contain protein. In contrast, many harmless powders often used as hoaxes do not contain protein. The presence of protein in a sample could indicate the presence of a biological agent. Conversely, the absence of protein would indicate that a biological agent is not present. In this step, commercially available protein detection test strips (designed, optimized, and used for protein detection in urine samples) are used to detect the presence of protein in a sample.

Taken together, the observations made using the five-step method were proposed to allow the first responder to determine if a material is most likely not a biological agent.

#### 4. METHOD EVALUATION

To evaluate the effectiveness of the five-step method for pre-screening suspicious white powders, an initial market study was conducted to identify inexpensive, commercially available components (urine test strips, pH strips, etc.) that could be used to perform the method. The study included those protein test strips that were inexpensive and easy to use. Forty-five various types of urine test strips were identified, but only six types of strips were ultimately included in the laboratory portion of the study. Protein strips with a number of unnecessary features (e.g., detection of bilirubin, ketones, blood, etc.) were not considered for fear that additional, extraneous information could confuse users. The field study only evaluated the two protein strips that performed the best in the laboratory study.

After the component materials were selected, blind testing of the five-step method was conducted in the laboratory by three independent evaluators. Blind tests were run on samples containing powdered forms of non-infectious strains of *Bacillus anthracis* (Sterne), the causative agent of anthrax, and *Yersinia pestis* (A1122), the bacteria that is responsible for bubonic plague. In addition, the 14 powders that comprise the suspicious powder panel, available from the Department of Defense's Critical Reagents Program (CRP), were tested (Table 2). To assess matrix interference, the study analyzed spiked samples containing the 14 powders from the CRP program spiked with the non-infectious *B. anthracis* spore powder and *Y. pestis* powders. A positive and negative control was used throughout the testing. In summary, the laboratory study consisted of five types of samples:

- Pure agent - unmixed non-infectious *B. anthracis* spore powder and *Y. pestis* powder
- Fourteen powders from the CRP suspicious powders panel
- Fourteen powders from the CRP suspicious powders panel spiked with avirulent *B. anthracis* spore powder or *Y. pestis* powder
- Positive Control
- Negative Control

A summary of the procedures used to analyze the samples using the five-step method are below:

1. Using a clean metal spatula 20 mg of each sample (pure agent, suspicious powders, spiked suspicious powders) were weighed, transferred to a clean vial, and randomly labeled prior to testing.

2. The vials were shaken vigorously for 5 s and observed by the evaluator for whether the powder formed a cloud/haze or fell to the bottom.

3. Two milliliters of sterile distilled water was added to the vial, the solution was shaken vigorously for 15 s, and the turbidity of the solution was recorded.

4. Each of the commercially available pH test strips were dipped into the solution and scored according to manufacturer's instructions.

5. Each of the commercially available protein test strips were dipped into the solution and scored according to manufacturer's instructions.

Table 2. Summary of Results of Protein Detection in Laboratory Study

Sample Type	Spike Type	Contains Protein?	% positive	% negative
Negative Control			0%	100%
Positive Control		✓	100%	0%
<i>B. anthracis</i>		✓	100%	0%
<i>Y. pestis</i>		✓	100%	0%
Powder 1			0%	100%
Powder 1	Spike 1	✓	100%	0%
Powder 1	Spike 2	✓	94%	6%
Powder 2			0%	100%
Powder 2	Spike 1	✓	100%	0%
Powder 2	Spike 2	✓	94%	6%
Powder 3			67%	33%
Powder 3	Spike 1	✓	100%	0%
Powder 3	Spike 2	✓	94%	6%
Powder 4			0%	100%
Powder 4	Spike 1	✓	94%	6%
Powder 4	Spike 2	✓	78%	22%
Powder 5		✓	33%	67%
Powder 5	Spike 1	✓	100%	0%
Powder 5	Spike 2	✓	100%	0%
Powder 6			0%	100%
Powder 6	Spike 1	✓	67%	33%
Powder 6	Spike 2	✓	33%	67%

Table 2. Summary of Results of Protein Detection in Laboratory Study (Continued)

Sample Type	Spike Type	Contains Protein?	% positive	% negative
Powder 7		✓	78%	22%
Powder 7	Spike 1	✓	100%	0%
Powder 7	Spike 2	✓	100%	0%
Powder 8			0%	100%
Powder 8	Spike 1	✓	100%	0%
Powder 8	Spike 2	✓	94%	6%
Powder 9		✓	89%	11%
Powder 9	Spike 1	✓	100%	0%
Powder 9	Spike 2	✓	94%	6%
Powder 10		✓	100%	0%
Powder 10	Spike 1	✓	100%	0%
Powder 10	Spike 2	✓	100%	0%
Powder 11			0%	100%
Powder 11	Spike 1	✓	94%	6%
Powder 11	Spike 2	✓	50%	50%
Powder 12			0%	100%
Powder 12	Spike 1	✓	94%	6%
Powder 12	Spike 2	✓	83%	17%
Powder 13			0%	100%
Powder 13	Spike 1	✓	100%	0%
Powder 13	Spike 2	✓	83%	17%
Powder 14		✓	100%	0%
Powder 14	Spike 1	✓	100%	0%
Powder 14	Spike 2	✓	94%	6%

After the initial laboratory study, a follow-on-study was conducted to evaluate the performance of the test in the field. The field testing, held at the Center for Domestic Preparedness (Anniston, AL), was conducted by 60 hazardous materials technicians dressed in Level-C personal protective equipment (PPE). The field tests only employed the 14 suspicious powders without the addition of any biological agents. Only two protein detection strips were evaluated during the field testing. The HAZMAT technicians performed the five-step method using the same general procedures as the laboratory test.

## 5. RESULTS

The five-step method proposes that measurements of particle size, solubility, pH, and protein content can reliably predict the absence of a biological threat agent in an unknown sample. Specifically, the method predicts that non-biological materials will have low turbidity in water, a pH <5 or a pH >9, and will not contain protein. In addition, the particle size of the sample should help the first responder determine whether the sample is an inhalation threat. Table 3 summarizes the predicted and actual observed particle size, turbidity, and pH results from the laboratory and field tests.

Table 3. Predicted and Actual Characteristics of the Biological and Hoax Powders

	Prediction	Actual
<b>STEP 2: Visual characteristics</b>		
Biological powder	Sticky	Sticky
Hoax powder	Non-sticky	Sticky
<b>STEP 3: Characteristics in water</b>		
Biological powder	Turbid	Turbid
Hoax powder	Non-turbid	Turbid
<b>STEP 4: pH</b>		
Biological powder	pH 5-9	pH 5-9
Hoax powder	pH<5 9<pH	pH 5-9

In the laboratory and field tests, the particle size of the samples containing biological material and those without biological materials were not easy to distinguish. All of the samples were observed as "sticky" and clung to the sides of the vials. In a similar manner, the method predicts that non-biological samples would dissolve in water and exhibit low turbidity, yet all of the samples resulted in a turbid solution when mixed with water. In addition, the expectation was that solutions with biological agents would have a neutral pH value (between 5 and 9), whereas non-biological powders could result in a solution that was more acidic with a pH value of <5 or more basic with a pH value >9. The results of the study revealed that all the samples had pH values between 5 and 9.

The results of the protein detection are somewhat more complex. The presence of protein should be positive for all samples containing biological components, which includes the positive control, pure agent powders, and powders spiked with agents. In addition, some of the suspicious powders, such as Dipel, flour, powdered milk, powdered coffee creamer, and yeast, naturally contain a biological component and would expect to be positive for protein. All other positive protein tests should be considered false positives. Additionally (and of more concern), samples containing biological agents that tested negative for protein should be considered false negatives. Table 2 summarizes the results of the protein detection tests in the laboratory study.

Overall, the protein detection test led to a 7% false positive rate and 11% false negative rate in the laboratory studies. The protein detection test was consistent with predictions for the pure agent powders, as well as for most of the hoax powders alone. However, one of the hoax powders (baking soda) consistently produced false positives. Also, a number of the spiked samples (those containing *B. anthracis* and *Y. pestis*) did not test positive for protein, which led to the fairly high number of false negatives.

The field-test results were consistent with the laboratory results described above. In general, particle size, turbidity, and pH were similar for samples containing biological components versus those without biological components. The PPE did not affect method performance. The field-testing did identify the need for a magnifying glass to be included in the kit components. In addition, training was identified as crucial to the success of the method given the difficulty in reading the color changes on the pH and protein strips.

## 6. DISCUSSION

Evaluation of the five-step method in the present study revealed strengths and weaknesses of the protocol, as well as with the commercially available components tested. In general, the proposed method was inexpensive, fast, easy to perform, and well-liked by the first responder community. However, multiple limitations of the method were discovered through this study and are discussed below.

The amount of material necessary to collect to ensure adequate detection of protein in the sample was found to be the size of a small pea. Unfortunately, the actual sample size at the incident site may not always be large enough to allow for the proper amount to be set aside for the LRN and for the pea size amount needed to perform the five-step method.

Also, the particle size, turbidity, and pH measurements were not useful for discriminating between biological and non-biological samples. With regards to particle size, all of the samples stuck to the sides of the container and made it difficult to observe a fine haze in the vial. It should be noted that no effort was made to size the particles in these powders or mix in any fluidizing agent. The solubility/turbidity step



also resulted in little distinction between biological and non-biological as all of the water samples were turbid. Perhaps most surprisingly, all of the samples (biological and hoax powders) were found to have a pH between 5 and 9. A possible problem with this test step is that the pH of distilled water in equilibrium with atmospheric carbon dioxide is 5.6 and the sample pH seems to reflect the pH of the water used and not of the sample itself. This lack of distinction between the results of the biological material and the hoax powder renders Steps 2, 3, and 4 useless for the purpose of ascertaining the probability that a sample is a non-biological.

The difficulties in Step 5 arose from weaknesses in the components, namely the protein test strips. The protein strips tested were able to detect protein in samples of pure *B. anthracis* spores and *Y. pestis*, as well as in suspicious white powders that did contain protein such as powdered milk, powdered coffee creamer, flour, Dipel, and yeast. However, one of the hoax powders that does not contain protein, baking soda, also had a positive reading (thus a false positive). This false positive could possibly be due to the baking soda interfering with the colorimetric chemistry of the test strips. However, an argument can be made that some false positives are acceptable for a pre-screening method as subsequent confirmatory testing would reveal the true nature of the sample.

Of most concern was the fact that the protein detection strips did not detect the presence of *B. anthracis* spores or *Y. pestis* when mixed with certain powders, which resulted in a number of false negative readings. Even though primarily there was little matrix interference observed, there were several circumstances when there was a significant masking effect that precluded the accurate detection of the biological threat agent. Whereas false positive samples can be ruled out through further testing, false negative readings could have much more serious consequences, allowing samples that are truly hazardous to pass undetected, posing a threat to public health.

The protein test strips used in this study were commercially-available urine test strips, which are designed to detect the presence of protein in urine and were never intended to be used for protein detection in any other material. In addition, reading the colorimetric change on the protein strips can be challenging, leaving room for human error. The results above show that these protein test steps can give false positive readings as well as, and more importantly, false negative readings, suggesting that this technology is not well suited for this application.

## 7. CONCLUSIONS

First responders frequently come into contact with suspicious white powders that are suspected to be biological threats, such as *Bacillus anthracis*. Due to the large number of suspicious white powder samples that first responders must handle, a need exists for an inexpensive, effective tool for pre-screening suspicious powders to identify hoax powders that do not need to be processed by expensive technologies/assays. The present study evaluated a recently proposed economical five-step pre-screening method that could be used to rule out suspicious white powders as biological

threats. The five-step method called for using commercially available test strips for determining pH and protein content of a sample as indicators of the presence of biological agent. To evaluate the five-step method, a blind study was conducted in which various protein and pH strips were used to analyze multiple samples of powder.

The amount of powder required to achieve detectable levels of bacteria amounted to a pea-sized portion of powders that was dispensed into sterile vials. In 2002, a similar study of biological agent detection in suspicious powders was conducted using a commercially-available FIRSTCHECK kit by 20/20 GeneSystems.<sup>2</sup> This test kit uses a swab-based protein detection and pH detection technology in contrast to the urine and pH test strips used in the proposed five-step method. The results of the 2002 study demonstrated that the swab-based technology could detect protein in samples of *Bacillus thuriengensis*, a spore forming bacteria related to *Bacillus anthracis*, in spore concentrations ten to one hundred fold less than detectable with the protein strips used in this study, which suggests that protein test strips are not the most sensitive technology available.

Furthermore, given the above results, a first responder following Steps 2 through 4 of the proposed five-step method would find all samples of suspicious powder to be possible biological agents because the characteristics of the biological agents and hoax powders were the same. Therefore, the true discriminating power of the method depends completely on Step 5 of detecting the presence of protein in the powder. However, the results above show that the protein test strips may not be sensitive enough and can give false positive readings, as well as false negative readings. Hence, using the method as it is currently designed may not be better than having to analyze all suspicious powder samples with the expensive, time-consuming assays to accurately identify the sample. Based on the outcome of these studies, it can be concluded that Steps 1 through 4 of the five-step method are ineffective at aiding in the differentiation of a true threat from a hoax, and Step 5 is not adequately reliable for use during a suspicious powder incident.

## 8. RECOMMENDATIONS

The lower sensitivity and high false negative rate of the protein test strips, in addition to the ineffectiveness of the other steps of the methods in discerning between biological agent and hoax powders, make it impossible to recommend the five-step method for use by first responders in the current format. The concept of using pH and protein detection technologies is certainly a valid approach for triage of suspicious white powders, however, the current commercially available test strip technology is not suitable for use in this test. Other technologies such as the 20/20 GeneSystems FIRSTCHECK kit may be a better alternative in the short term, given its

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<sup>2</sup>Kronenberg, J.; Lorch, D. Summary Report of 20/20 Gene Systems Inc. on Bio-Identification Kit Testing: Tier I and II. Battelle, 2002

higher protein sensitivity. However, this study did not test that system and no claims can yet be made as to its effectiveness.<sup>3</sup> Further research is required and is currently underway to develop a cost-effective method that will enable first responders to accurately pre-screen incidents involving suspicious white powders.

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<sup>3</sup>Marcus, Liz. Letters. *Homeland First Response*. November/December 2003; 9.